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(54) FUSION PROTEINS WITH REPRESENTATION OF DIFFERENT ALLERGENS: VACCINE PROPOSAL FOR MITE ALLERGIES

(57) The present invention refers to recombinant DNA molecules codifying fused peptides from different allergens from Blomia tropicalis and Dermatophagoides pteronyssinus having potential usefulness in prevention and treatment of allergies caused by domestic mites. Specifically, the invention discloses fusion proteins composed by different fragments of allergens Der p 1, Der p 2, Der p 7, Der p 8,Blo t 5, Blo t 8, Blo t 18, Blo t 12 and Blo t 13 with reduced serum IgE reactivity in allergic and non allergic individuals. It also discloses methods for production of these molecules in an expression system based on E. coli and purification. The invention refers also to effective and safe vaccines.

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Description

TECHNICAL FIELD

[0001] The present invention relates to design of synthetic nucleotide sequences and to production of recombinant fusion proteins for treatment and prevention of allergies caused by domestic mites, in particular those caused by species Blomia tropicalis and Dermatophagoides pteronyssinus.

BACKGROUND OF THE INVENTION

[0002] Individuals allergic to domestic mites are in general sensitive to mites of genus Dermatophagoides, being D. pteronyssiunsand D. farinae the most prevalent species worldwide. Additionally, in tropical and sub-tropical regions the presence of B. tropicalis is very abundant, being predominant sometimes in the population of mites present in house dust. In these regions, individuals allergic to mites are sensitive mostly to D. pteronyssinus and B. tropicalis (1).

[0003] About twenty allergens have been identified among domestic mites. However, only some of those have shown high reactivity frequency in the allergic population and some show cross-reactivity. Amongst B. tropicalis allergens, Blo t5 shows the highest reactivity frequency, Blo t12 and Blot 13 show lower frequency, but induce an intense IgE in many of the sensitive individuals (2, 3).

[0004] Others as Der p1 and Der p2 are the most important D. Pteronyssinus, and between 50% and 100% of allergic individuals have IgE specific against these two allergens.

[0005] Another D. Pteronyssinus important allergen is Der p7, being estimated that 50% of individuals allergic to domestic mites shows IgE against this allergen. Allergens of mite groups 8 and 10, such as glutatione S transferase (GST) and tropomyosine, respectively, are of particular importance due to the significant crossed-reactivity observed with homologous derivate from different mite species and other organisms (4).

[0006] An allergen must produce cross-linking of IgE antibodies linked to the effector cells surface in order to induce cell activation and inflammatory response, requiring this process at least two IgE epitopes on the allergen surface. The IgE antibodies of an allergic patient may recognize continuous epitopes or discontinuous epitopes. Cross-linking of IgE antibodies on mast cells surface induce immediate release of biologically active mediators such as histamine and leucotrienes.

[0007] Decrease of allergenic capability can be achieved by intervention of IgE epitopes through mechanisms such as point mutation, amino acid deletion and disturbance of the natural molecular bending. Reordering amino acid segments conforming the primary sequence of the allergen also modifies the allergenic capability.

[0008] In some studies fusion or hybrid proteins have been generated to provide a better allergen-specific immunotherapy. Fusion proteins have been generated by using several portions of different allergens linked to form a unique molecule, intended to obtain vaccines poorly reactive to IgE antibody, which decreases the anaphylaxis risks, and to deliver to the patient only those allergens involved in development of allergic response (5).

[0009] A number of patents is known in this field related to pollen allergens (US7,862,828) and to domestic mites, as shown in documents US20080274059; US20110052640; US20070065468; US20060233839; US20090130130 and WO2007140505.

[0010] In fact, document US 7,862,828 describes a method to prepare fusion allergens, consisting of two or more recombinant polypeptides from grass pollen, to be used as immunotherapeutic agent comprising: (a) to provide a polynucleotide sequence which codifies the fusion allergen, (b) to introduce said polynucleotide sequence into the host cell, (c) to grow the host cell to express the sequence, (d) to recover the fusion allergen expressed from the grown cell, (e) to assay the fusion allergen as a candidate for immunotherapy, by delivering the allergen to a test animal and to select those inducing the strongest immune response and generation of IgE blocking antibodies in comparison to that obtained with the individual components.

[0011] On this case, the polypeptide has at least two different allergenic proteins or their fragments. The hybrid polypeptide, the polynucleotide and the cell are useful for drug preparation for treatment of allergenic disorders or for prophylactic vaccination. Among allergenic sources for allergenic proteins, grass pollen, mites, bee venom or animal hair allergens may be the most important. Specific examples of allergenic proteins are group 1, group 2, group 4, group 5, group 6, group 11, group 12 and group 13 of allergens from the most important grass pollen, Der p 1 and Der p 2 (mites), phospholipase from bee venom, and Fel d 1 (cat).

[0012] On the other hand, document US20080274059 reveals fusion protein comprising a group 1 allergen and a group 2 allergen from genus Dermatophagoides fused at group 1 allergen N- o C-, where they may be Der p 1 or proDer p 1, and, from group 2, where they may be Der p 2, This document includes claims for the nucleic acid to develop the fusion protein, the expression cassette, the vector containing this cassette and a cell of any type including cassette or expression vector, and a vaccine composition including the fusion protein on a pharmaceutically acceptable support. The fusion protein is applied to prevent or treat allergic reactions to mites. The expression cell may be Escherichia coli,

Pichia pastoris or Saccharomyces cerevisiae.

[0013] The United States Patent Application 20110052640 reports a hypoallergenic hybrid polypeptide comprising an amino acid sequence of at least 50 amino acids of a sequence selected from group 1 and 2 allergens from dust mites (Dermatophagoides pteronyssinus), and where a linkage epitope to IgE antibody of said allergen is deleted. Included are either the polypeptide having 70% sequence identity, the corresponding polynucleotide, the vector, the expression host cell including the polynucleotide, the method to produce the polypeptide and the pharmaceutical composition that comprises the polypeptide. The group 1 allergen is Der p 1, and that of group 2 is Der p 2. The obtained polypeptide keeps immunogenic capabilities, being particularly useful for allergy treatment. Additionally, production methods for these polypeptides in heterologous expression systems and efficient purification methods are described

[0014] Patent US20070065468 reports a product for specific reduction of immunoglobulin E (IgE) as well as the allergic reaction, specifically a chimeric polypeptide having at least two mite allergens. It includes amino acid sequence for those allergens such as Der p1, Der p 2 and Blo t 5. Allergens can be expressed in Escherichia coli or CHO-K1.

[0015] Patent US20060233839 describes a recombinant protein with the allergen derivate from Der p1 where the protein has a reduced allergenic activity, important in relation to the allergen in natural state and comprising three mutation sites. Additionally to Der p 1 allergen, ProDer p 1 and Der p 3 with ProDer p 3 y PreProDer p 3 are included. Claims include the nucleic acid molecule, the expression vector, the cell transformed for production of recombinant protein, and the immunological composition used for patient treatment.

[0016] Document US20090130130 refers to an isolated polypeptide, derivative or fragment composed by (a) a polypeptide, its derivate isoform, or a fragment thereof, comprising a non-helicoidal mutant of at least an allergen from group 5 mites and, (b) a polypeptide, its derivate isoform, or a fragment thereof comprising at least a substituted, added or deleted amino acid, or at least a chemical modification, where the polypeptide exhibits reduction in reactivity equal to or higher than IgE from the natural polypeptide, in subjects allergic to at least one allergen from group 5 mites. This polypeptide from the group 5 allergens may be Blo t 5, Der p 5, Der f 5, or Der m 5. Claims include nucleic acids sequences, a vector/host cell comprising nucleic acid molecules, a pharmaceutical preparation including the polypeptide or a vaccine.

[0017] Patent WO2007140505 reports a hypoallergenic protein comprising an allergen fused to or conjugated with at least a second non-allergenic protein or a fragment thereof. Included are claims for nucleic acid molecules codifying for the hypoallergenic molecule or the fused protein, the vector, the host cell, an antibody addressed against the hypoallergenic or fusion protein, and the vaccine which may comprise it. The protein is derivate from Phl p 5, Fel d 1, Der p 2, Der p 7, Derp 21, Clone 30, Alt a 1, Par j 1, Ole e 1, Fel d 2, Can f 1, Can f 1, Art v 1, Amb a1, Alt a 2 or Alt a 6 having a terminal blunt in C- o N-, and exhibiting reduced capability to IgE in comparison to native Phl p 5. The second protein is a viral, bacterial, fungal, or protozoan protein. For example, it is a virus capside protein, preferably from picornaviridae.

[0018] Additionally, it is known that Linhart y collaborators (6) built, by PCR-based recombination, a fusion molecule composed by the most important allergens from timothy grass, in the order Phl p6, Phl p2,Phl p5 y Phl p1, which was expressed in E. coli as a 79 kDa protein. This molecule demonstrated to be useful for immunotherapy since it induced limpho proliferation in a degree similar to that induced by a equimolar mix of the individual allergens in an model of mice allergic to pollen, induced Th1 and IgG profile cytokine production, able to block degranulation of mast cells incubated in the presence of the allergen.

[0019] The concept of fusion proteins has been also explored with mite allergens; a fusion protein composed by Der p1 and Der p2 portions showed lower IgE reactivity in comparison to native allergens and may induce mice to produce blocking IgG, which suggests their usefulness in immunotherapy (7).

[0020] Recently, Bussieres L. and collaborators (8) built several hybrid proteins composed by Der p1, Der p2 portions and a Der p1 precursor (pro-Der p1), showing that they are able to induce basophile degranulation in individuals allergic to domestic mites, and by "immunoblotting" with polyclonal and monoclonal antibodies, the conservation of B epitopes of native allergens was demonstrated.

[0021] Nevertheless, there is a non-satisfied need for vaccines based on recombinant molecules that contain several antigenic regions from mites common in tropical and subtropical areas, which may be suitable alternatives to immunotherapy schemes currently known, which are based on application of natural extracts from complete allergens, and which may be able to decrease the anaphylaxis risk and to improve the treatment efficacy.

DEFINITIONS

[0022] The terms used herein will have the following meanings:

"allergen", a substance able to induce an allergic reaction by inducing production of IgE antibodies.

"vaccine", a substance which can be used to stimulate the immune system to develop protection; the substance may be a polypeptide, such as the fusion protein subject of the present invention, in presence or absence of pharmaceutically acceptable carriers or vehicles.

"expression", refers to a gene or a gene product expression including the codified polypeptide.

DESCRIPTION OF THE DRAWINGS

[0023]

FIG 1. Schematic representation of peptides conforming the primary sequence of recombinant PF3a.

FIG 2. Levels of serum IgE specific for PF14c determined by ELISA, in comparison to reactivity levels of natural extracts from B. tropicalis and D. pteronyssinus.

FIG 3. Schematic representation of peptides conforming the primary sequence of recombinant PF14c.

FIG 4. Levels of serum IgE specific for PF14c determined by ELISA in comparison to reactivity levels of natural extracts from B. tropicalis and D. pteronyssinus.

FIG 5. Purification profile for PF3a performed by gel electrophoresis on 15% polyacrylamide, stained with Comassie Blue. 1 is the molecular weight marker, 2 and 3 correspond to non-dialyzed samples, and 4 corresponds to the dialyzed sample in 0.1% Triton X-100 buffer.

FIG 66. Purification profile for PF14c performed by gel electrophoresis on 15% polyacrylamide, stained with Comassie Blue. 1 is the molecular weight marker, 2 y 3 represent the purified fractions.

DETAILED DESCRIPTION OF THE INVENTION

[0024] The present invention is directed to fusion proteins with different allergen epitopes and reduced linking capability to IgE. The present invention is also directed to a pharmaceutical presentation allowing application within a cutaneous vaccination scheme ("Skin PrickTest"). The composition is: 1. Fusion protein, 2. Glycerol, and 3. Phenol.

[0025] The invention also reports synthetic nucleotide sequences design and production of recombinant fusion proteins for treatment and prevention of allergies caused by domestic mites, particularly caused by species Blomia tropicalis and Dermatophagoides pteronyssinus.

[0026] In other embodiment, the invention comprises nucleotide sequences artificially designed, codifying proteins related to domestic mites allergens.

[0027] The invention also refers to the design of two vectors based on vector pET45b+, the artificially designed nucleotide sequence being inserted such that a suitable reading frame is preserved, which expresses the recombinant protein with a histidine 6X tag, in the amino terminal region.

[0028] In this sense, the present invention is also addressed to vector pET-45b(+) which is a plasmid vector specifically ordered to Novagen, having a sequence that codifies by means of a histidine 6x tag in the N-terminal region, followed by a sequence for a cleavage site by means of enzyme enterokinase and a sequence for a S-tag in the C-terminal region. It contains 5260 base pairs. The designed nucleotide sequence insertion was ordered for the fusion protein, PF3ao PF14c, at the position flanked by restriction sites Pml 1 and Kpn I, and such that a reading frame (ORF- Open ReadingFrame) was generated to express the protein of interest with nine additional amino acids (MAHHHHHHV) in the N-terminal region under control of promoter T7. In this way, the invention reports a vector referred as pET45b/PF3a constituted by 5852 base pairs and a vector referred as pET45b/PF14c constituted by 5719 base pairs, which, when introduced into E. Coli bacteria, allow expression of recombinant proteins PF3a having 196 amino acids (SEQ ID No. 2) and PF14 c having 152 amino acids (SEQ ID No. 4), exhibiting the unique features disclosed in the present invention.

[0029] The invention also contemplates the possibility to combine, in a sole composition, the pharmaceutical formulation or vaccine including the two recombinant proteins making possible to improve or enhance their efficacy in immunotherapy against domestic mite allergy.

[0030] The vaccine for immunotherapy against domestic mite allergy according to the present invention is characterized by an-amino acid sequence selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 4 or a mix thereof.

[0031] In still another embodiment, the invention refers to a method to obtain a recombinant fusion protein of different allergen segments from B.tropicalis expressed in Escherichia coli, which shows reactivity against IgE antibodies of an allergic population with lower frequency and intensity in comparison to that obtained from the allergenic extract from B. tropicalis.

[0032] Even another embodiment of the invention refers to a method to obtain a recombinant fusion protein having several allergen segments from B. tropicalis and D. Pteronyssinus, expressed in Escherichia coli, which shows reactivity against IeG antibodies of an allergic population with lower frequency and intensity in comparison to that obtained from

the corresponding natural extracts.

[0033] The invention also refers to use of proteins designed according to the invention to prepare a medicament vaccine to treat an individual suffering allergies caused by domestic mites.

[0034] The invention is also addressed to a treatment method for an individual suffering allergies caused by domestic mites, which comprises administering to said individual a pharmaceutically effective amount of a protein according to the invention or a pharmaceutical composition containing the same.

[0035] Protein design according to the present invention starts with the selection of different allergens from domestic dust mites B. tropicalis and D. pteronyssinus, as well as of their antigen regions as reported in scientific publications or predicted by our bioinformatic analysis. The different selected regions were linked to form a unique primary protein structure. The probable 3D-structure for the designed protein was modeled from the amino acid sequence. The structure was optimized targeting to a successful expression as recombinant protein. In this manner, respective models were obtained for each of the two fusion proteins subject of the present invention.

[0036] Prediction for three-dimensional structure was performed by using I-TASSER (9) and Swiss-Model tools and software. (10, 11). Segments combination and order producing a model satisfactory with regard to energy, stereochemistry and structure requirements were selected to order synthesis of an artificial nucleotide sequence codifying for the primary sequence.

[0037] One vector was chosen for expression in E. Coli, i.e., pET 45b+ (NovagenR, catalog 71327-3), which was inserted in the artificial sequence to obtain a reading frame for expression of the codified protein at the insertion site, with a histidine 6x tag in the N-terminal region, with no cleavage site to remove the tag.

[0038] The artificial gene synthesis with codon optimization for expression in bacteria, and its insertion in vector pET 45b+, such that a vector pET 45+b/PF was obtained for expression of fusion protein linked to the histidine 6x tag, was ordered to a US specialized company (GeneScript).

[0039] Once the artificial gen and the expression vector with insert were received, transformation was initiated in different E. Coli strains, according to the requirements of the protein of interest. The positive clones were selected after confirmation of the expected insert by PCR using universal primers, and expression was performed in LB (Luria-Bertani) medium, consistent of 1% tryptone, 0,5% yeast extract y 1% NaCl, pH 7.0, with antibiotic, IPTG (isopropyl-β-D-thiogalactoside) induction, one lactose analog, which acts as a protein expression regulator under lac operon control. Then, the culture was centrifuged for 10 minutes at 4000 - 6000 r.p.m., and the cell pellet was recovered. A standard procedure was followed (The Condensed Protocols from Molecular Cloning: A Laboratory Manual, Sambrook and Russel, CSHL-Press), with modifications.

[0040] The cell pellet obtained was re-suspended in lysis buffer (NaH2PO4 100mM, Tris-HCl 10 mM, Urea 8 M, pH 8.0) and stirred at 20 r.p.m. from one to two hours. The mixture was centrifuged 6000 r.p.m. for 10 minutes at 4°C , and the soluble fraction contained in the supernatant was recovered. 8 ml of this supernatantwere mixed with Ni-NTA resin previously prepared by washing twice with water and balanced with lysis buffer, pH 6, and was stirred at 20 r.p.m. for two hours. The mixture was centrifuged at1200-1600 r.p.m. for one minute, and the supernatant containing the fraction not linked to the resin was separated. The resin was washed several times with NaH2PO4 20 mM, Urea 8 My NaCl 500 mM buffer solution at pH 8.0, 6.0 y 5.3 (2 washings at every pH). After every washing, the fraction not linked to the resin was separated by centrifugation at 1200-1600 r.p.m. for one minute and then discarded. The resin was loaded on a column and the fusion protein was recovered by application of a buffer elution solution (NaH2PO4 20 mM, Urea 8 M, NaCl 500 mM at pH 4.0) to the column. Presence of the band of interest in the collected fractions was analyzed by electrophoresis on 15% poly-acrylamide under reductive conditions. Fractions were combined according to the intensity of the band of interest on the electrophoresis gel, and were diluted with the buffer elution solution.

[0041] To eliminate urea and induce protein re-naturalization, arginine-assisted oxidative re-naturalization was performed. In summary: fractions obtained and diluted after purification were dialyzed in dialysis bags having a 6-8000 kDa limit, against re-naturalization solution (100 mM Tris-HCl, 10 mM EDTA, 0,5 M L-Arginine, 5 mM Cysteine, 1 mM Cystine) at 1:20 ratio, under moderate and constant stirring overnight at 4°C. Then, dialysis was performed against 10 mM Tris-HCl, 0,1% Triton X-100 buffer solution for 4 hours with exchange at 2 hours. The re-naturalization products were analyzed by electrophoresis on 15% polyacrylamide under reduction conditions, and sorted at -20°C.

[0042] The linking capability of IgE and IgG antibodies on human sera was demonstrated by ELISA, electrotransfer, and immunodetection techniques, as per a procedure standardized in our laboratory.

[0043] The method disclosed in the present invention to obtain a recombinant protein from allergen segments of B. tropicalis (PF14c) and allergen segments of B. tropicalis y D. pteronyssinus (PF3a) expressed in E. Coli, with reactivity against IgE antibodies in allergic populations, with lower frequency and intensity in comparison to natural allergenic extracts from B. tropicalis y D. Pteronyssinus, comprises the following steps:

Transformation of E. Coli cells:

[0044] Vector pET 45b(+)/PF containing the fusion protein segment of codifying nucleotides was used to transform

competent One Shot® BL21StarTM (DE3) (Invitrogen, Cat. No. C6010-03) y TOP10 (Invitrogen Cat. No. C4040-10) E. coli cells, by means of a chemical treatment combined with thermal shock. In summary, 10 ng of vector were mixed and incubated with competent cells for 30 minutes on ice, followed by 30 seconds at 42°C and then fast cooling on ice. To the foregoing mix, 250 μL of S.O.C optimized medium (consisting of 2% tryptone, 0,5% yeast extract, 10 mM NaCl, 2,5 mM KCl, 10 mM MgCl2, 20 mM glucose) were added. Then, the mix was incubated at 37°C, 250 r.p.m. for 30 minutes. The mix was grown on LBA plates (Luria-Bertani, 1% tryptone, 0,5% yeast extract, 1% NaCl, pH 7.0, con ampicillin 100 mg/mL) at 37°C overnight.

Selection of transformed colonies:

[0045] The presence of inserts in the transformed colonies was determined by PCR (Polymerase Chain Reaction) directly from the colonies, using the universal primers "T7 Promoter" (5'-TAATACGACTCACTATAGGG-3') y "T7 Terminator" (5'-TAGTTATTGCTCAGCGGTGG-3'). For PCR, a reaction mixture was prepared as follows:

DNA template (colony) 100 ng	2 μL
dNTPs 50mM	1 μL
Primer "T7 terminator" (10 pmol)	1 μL
Primer "T7 promoter" (10 pmol)	1 μL
Buffer solution for PCR 10X	5 μL
MgCL2 50 mM	1.5 μL
Taq Polymerase 5U/μL (Invitrogen, Cat. No. 116 15-010)	1 uL
Deionized water c.s.p.	50 μL

[0046] Amplification schedule consisted of 30 cycles in three steps as follows: 1) 94°C for one minute, 2) 50°C for one minute, and 3) 72°C for 2 minutes, with a 7-minute final extension at 72°C. The reaction was performed in a S-1000 (BioRad) thermocycler. The amplification product was analyzed by electrophoresis on 2% agarose on a Mini SubTM DNA Cell (BioRad) instrument at 50volts. Positive clones were stored at -70°C in 8% glycerol.

Expression of fusion protein:

[0047] The protein linked to the six-residue histidine tag (6xHis-tag) was obtained by induction with IPTG (isopropyl-β-D-thiogalactoside, a galactose analog, protein expression regulator under control of operon lac). Only one colony isolated from a culture of positive clone incubated on a LBA plate was grown on liquid LBA medium and incubated at 37°C, 250 r.p.m. for 12-14 hours. Then, the culture was diluted in liquid LBA (1:20 dilution) and incubated for 3-4 hours under same conditions until a OD600 (optical density at 600 nm) of 0.5-0.8 was accomplished, when IPTG was added and kept in induction for 3-6-hours at 37°C. Then, the culture was centrifuged for 10 minutes at 4000 - 6000 r.p.m. and the cell pellet was recovered. A standard procedure was followed (The Condensed Protocols from Molecular Cloning: ALaboratory Manual, Sambrook and Russel, CSHL Press), with modifications.

Purification and re-naturalization of fusion proteins:

[0048] The cell pellet obtained above was re-suspended in lysis buffer (100 mM NaH2PO4, mM Tris-HCl 10,8 M Urea, pH 8.0), stirred at 20 r.p.m. for 1-2 hours. The mix was centrifuged at 6000 r.p.m. for 10 minutes at 4°C, and the soluble fraction contained in the supernatant was recovered. 8 ml of this supernatant were mixed with the affinity resin based on Ni-NTA previously prepared by washing twice with water and balanced with lysis buffer, pH 6, and was stirred at 20 r.p.m. for two hours. The mixture was centrifuged at 1200-1600 r.p.m. for one minute, and the supernatant containing the fraction not linked to the resin was separated. The resin was washed several times with NaH2PO4 20 mM, Urea 8 M y NaCl 500 mM buffer solution at pH 8.0, 6.0 y 5.3 (2 washings at every pH). After every washing, the fraction not linked to the resin was separated by centrifugation at 1200-1600 r.p.m. for one minute and then discarded. The resin was loaded on a column and the fusion protein was recovered by application of a buffer elution solution (NaH2PO4 20 mM, Urea 8 M, NaCl 500 mM at pH 4.0) to the column. Presence of the band of interest in the collected fractions was analyzed by electrophoresis on 15% poly-acrylamide under reduction conditions. Fractions were combined according to the intensity of the band of interest on the electrophoresis gel, and were diluted with the buffer elution solution.

[0049] To eliminate urea and induce protein re-naturalization, arginine-assisted oxidative re-naturalization was performed. In summary: fractions obtained and diluted after purification were dialyzed in dialysis bags having a 6-8000 kDa limit, against re-naturalization solution (100 mM Tris-HCl, 10 mM EDTA, 0,5 M L-Arginine, 5 mM Cysteine, 1 mM Cystine)

at 1:20 ratio, under moderate and constant stirring overnight at 4°C. Then, dialysis was performed against 10 mM Tris -HCl, 0,1% Triton X-100 buffer solution for 4 hours with exchange at 2 hours. The re-naturalization products were analyzed by electrophoresis on 15% polyacrylamide under reduction conditions, and were stored at - 20°C.

Features of recombinant proteins:

PF3a:

[0050] It is the protein corresponding to SEQ ID No. 2 and comprises 196 amino acids corresponding to different fragments contained in allergens from mites Blomia tropicalis and D. petronyssinus; Blo t 5 (Accession Number:096870.1, GI:14423644and 2 JRKA GI:193506481), Der p 2 (Accession Number:P49278.1, GI:1352237), Blo t 10 (Accession Number ABU97466.1, GI:156938889),Der p 8 (Accession Number P46419.1, GI:11700§5), Blo t 8 (ACV04860.1,GI:256665455) Der p 1 (Accession Number ACG58378.1, GI:195933901), Der p 2(Accession Number P49278.1, GI:1352237 and Der p 7 (Accession Number 3H4Z-AGI:292659601). This protein with a 196 amino acid sequence, 22.8 KD,has allergenic segments from allergens from mites Blomia tropicalis and Dermatophagoides pteronyssinus.

PF14c:

[0051] This is the protein corresponding to SEQ ID No. 4, comprising 152 amino acids corresponding to different fragments found in 6 allergens from mite Blomia tropicalis, Blo t 5 (Accession Number: ABH06352.1, GI:111120436), Blo t 8 (ACV04860.1, GI: 256665455) Blo t 1,0 (Accession Number ABU97466.1, GI: 156938889), Blo t 12 (Q17282.1, GI: 2498195) y Blo t 13(Q17284.1, GI:14423698). This 152 amino acid protein, 17.8KD, has allergenic segments from allergens from mite Blomia tropicalis.

SEQUENCE LISTING

Sequence ID. No. 1 (PF3a)
Length: 592
Type: DNA
Organism: Artificial
Other Information: synthetic sequence

```
1   ATGGCACATC ACCACCACCA TCACGTGAAC AAGAGCAAGG AATTGCAAGA GAAAATCATT 60
61  CGAGAACTTG ATGTTGTTTG CGCCATGATC GAAGGAGCCC AAGGAGCTTT GGAACGTGAA 120
121 TTGAAGCGAA CTGATCTTAA CATTTTGGAA CGATTCAACT ACGAACAACG TGAGGAAGCT 180
181 TATGAGCAAC AAATCCGAAT GATGACCGGC AAGCTCAAGG ATTTTAATCT TTATGAATAT 240
241 TTGTGCCATG TCAAGGTGAT GGTGCCCGAA GTGTTCGGTG ATTTTAAAAT GACTGAGTCA 300
301 AGTAGCAATT TTGAAACGAT TAGACGCATT CCGTCATTAT GATGGCCGAA CAATCATTCA 360
361 ACGCGATGTT CCCGGTATCG ATCCAAATGC ATGCCATTAT ATGAAATGTC CATTGGTTAA 420
421 AGGACAACAA TATGATATTA AATATACATG GAATGTTCCA AAAATTGCAC CAAAATCTGA 480
481 AAATGTTGTC GTCACTAGTG AAGATGGTGT TGTCAAAGCT CATTTGTTGG TCGGTGTTCA 540
541 TGATGACGTT GTTTCAATGG AATATGATTT AGCATACAAA TTGGGTGATT AA          592
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Sequence ID. No. 2
Length: 196
Type: protein
Organism: Artificial
Other Information: Peptide fusion protein homologous to mite allergens

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1   MAHHHHHHVN KSKELQEKII RELDVVCAMI EGAQGALERE LKRTDLNILE RFNYEQREEA  60
61  YEQQIRMMTG KLKDFNLYEY LCHVKVMVPE VFGDFKMTES VAILKRLDAF RHYDGRTIIQ 120
121 RDVPGIDPNA CHYMKCPLVK GQQYDIKYTW NVPKIAPKSE NVVVTSEDGV VKAHLLVGVH 180
181 DDVVSMEYDL AYKLGD    196
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Sequence ID. No. 3
Length: 459
Type: DNA
Organism: Artificial
Other Information: synthetic sequence

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1   ATGGCACATC ACCACCACCA TCACGTGAAC AAGAGCAAGG AATTGCAAGA GAAAATCATT 60
61  CGAGAACTTG ATGTTGTTTG CGCCATGATC GAAGGAGCCC AAGGAGCTTT GGAACGTGAA 120
121 TTGAAGCGAA CTGATCTTAA CATTTTGGAA CGATTCAACT ACGAACGTCG TATTCAATTG 180
181 ATCGAAGAAG ATTTGGAACG ATCAGAAGAA CGACTTAAAA TTGCAACAGC TAAATTGGAA 240
241 GAAGCATCAC AATCTGCCAT AATATGTTCC AAATCTGGTT CATTGTGGTA CATTACCGTA 300
301 ATGCCATGTT CGATTGGAGA TTTTAAAATG ACTGAGTCAG TAGCAATTTT GAAACGACGT 360
361 AGTTTGAGTA CGTTCGAAGG TGACAACAAG TTTATTCACA CCGAACCCGA TGATCATCAC 420
421 GAAAAGCCAA CGACCCAGTG CACCCATGAG GAGACATGA                          459
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Sequence ID. No. 4 (PF 14c)
Length: 152
Type: protein
Organism: Artificial
Other Information: Peptide fusion protein homologous to mite allergens

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1   MAHHHHHHVN KSKELQEKII RELDVVCAMI EGAQGALERE LKRTDLNILE RFNYERRIQL  60
61  IEEDLERSEE RLKIATAKLE EASQSAIICS KSGSLWYITV MPCSIGDFKM TESVAILKRR 120
121 SLSTFEGDNK FIHTEPDDHH EKPTTQCTHE ET  152
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Claims

1. A nucleotide sequence artificially designed which codifies proteins representing different epitopes from several domestic mite allergens where said nucleotide sequence is selected from the group consisting of SEC ID N° 1 and

SEQ ID N° 3.

2. An amino acid sequence **characterized in that** the said sequence is SEQ ID N° 2 or SEQ ID N1 4 and **in that** said amino acid sequence is codified by the nucleotide sequence SEQ ID N° 1 and SEQ ID N° of Claim 1, respectively.

3. Design of a vector **characterized by** the insertion in sequence of vector pET45b+ of the nucleotide sequence selected from SEQ ID N° 1 and SEQ ID N° 3, such that an appropriate reading frame is preserved for expression of the recombinant protein with a histidine 6x tag, in the amino terminal region.

4. A vector **characterized in that** said vector contains a codifying sequence by means of a histidine 6x tag in the N-terminal region followed by a nucleotide sequence selected from the group consisting of sequences SQ ID N° 1 and SEQ ID N° 3 according to Claim 1.

5. The vector according to Claim 4, **characterized in that** said vector has 5852 base pairs including SEQ ID N° 1, which, when introduced in E. coli allows expression of recombinant protein of SEQ ID N° 2.

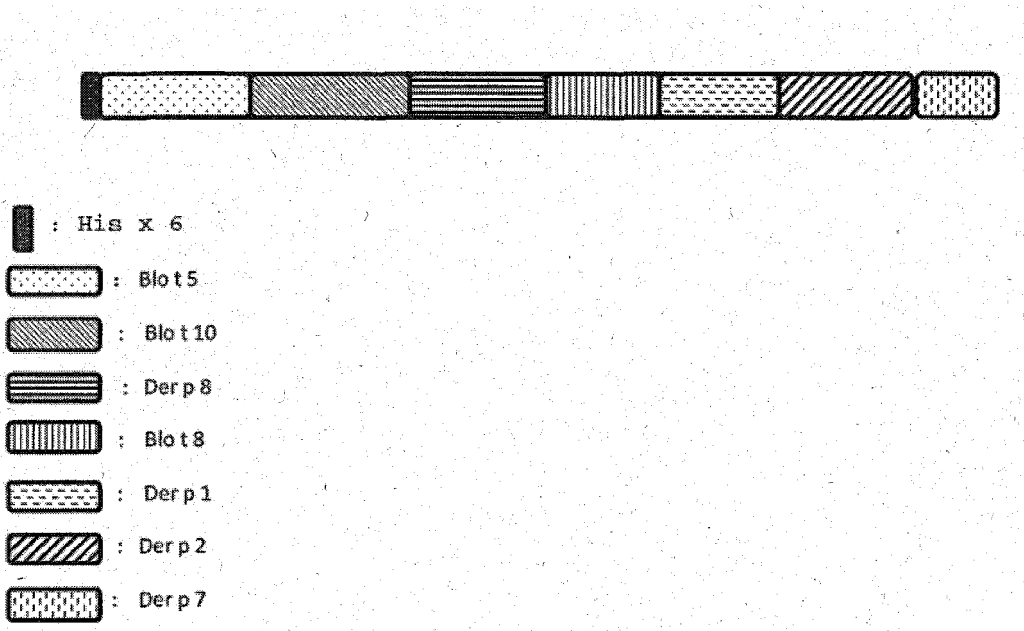
6. The vector according to Claim 4 **characterized in that** said vector has 5719 base pairs including SEQ ID N° 2, which, when introduced in E. coli allows expression of recombinant protein of SEQ ID N° 4.

7. A method to obtain a recombinant fusion protein of Claim 2 **characterized in that** said method comprises the following steps:

    a. transformation of E. coli competent cells having the vector, by means of chemical treatment combined with thermal shock;
    b. selection of transformed colonies by means of confirmation of the designed gene presence by PCR technique (polymerase chain reaction);
    c. expression of recombinant protein by cell growth with antibiotic induced by IPTG (isopropyl-p-D-thiogalacto-side);
    d. purification of the recombinant protein by affinity resin based on nickel after treatment with urea;
    e. re-naturalization of the recombinant protein by the arginine-assisted oxidative re-naturalization technique and dialysis.

8. A vaccine for domestic mite allergy immunotherapy **characterized in that** said vaccine contains an amino acid sequence selected from the group consisting of SEQ ID N° 2 and SEQ ID N° 4.

9. A vaccine for domestic mite allergy immunotherapy **characterized in that** said vaccine contains amino acid SEQ ID N° 2 and amino acid SEQ ID N° 4.



Primary sequence of recombinant PF3a

FIG. 1

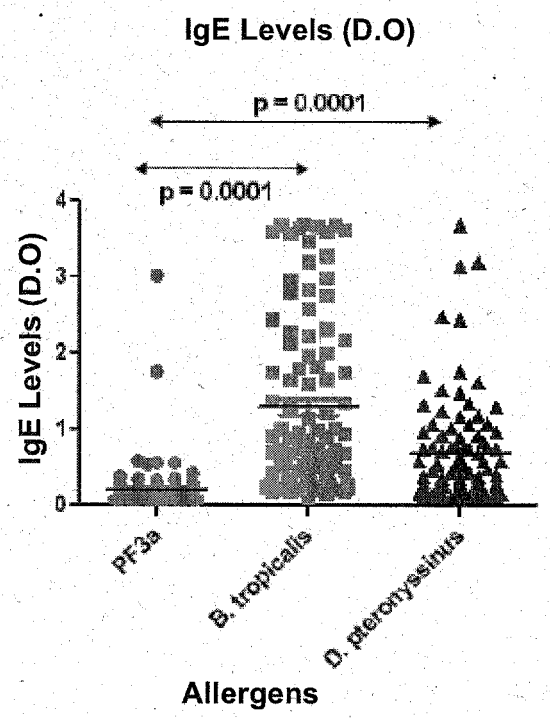
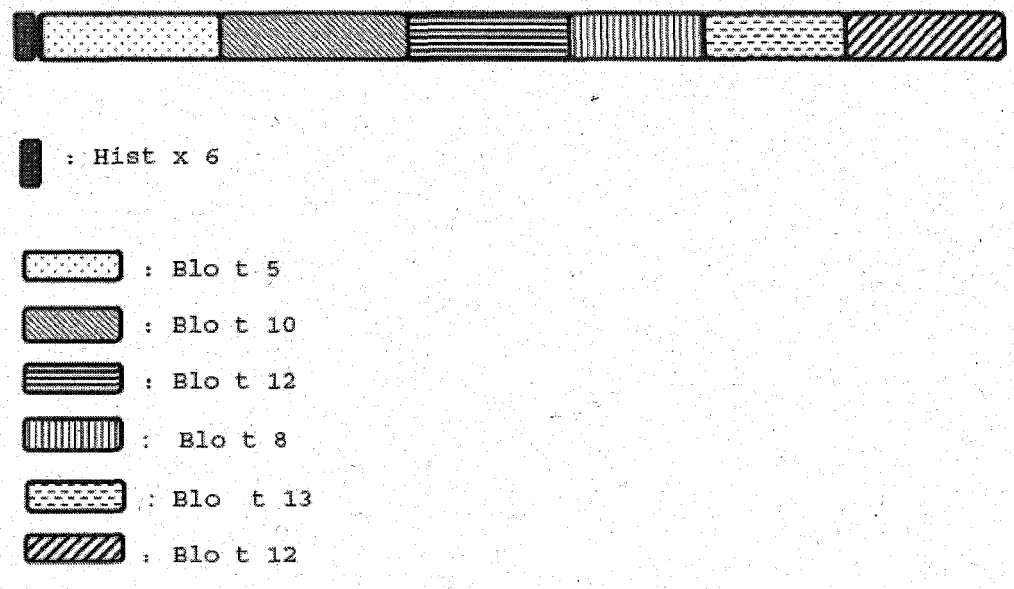

IgE Levels (D.O)
p = 0.0001
p = 0.0001
IgE Levels (D.O)
0
1
2
3
4
PF3a
B. tropicalis
D. pteronyssinus
Allergens

FIG. 2



Primary sequence of recombinant PF14c

FIG. 3

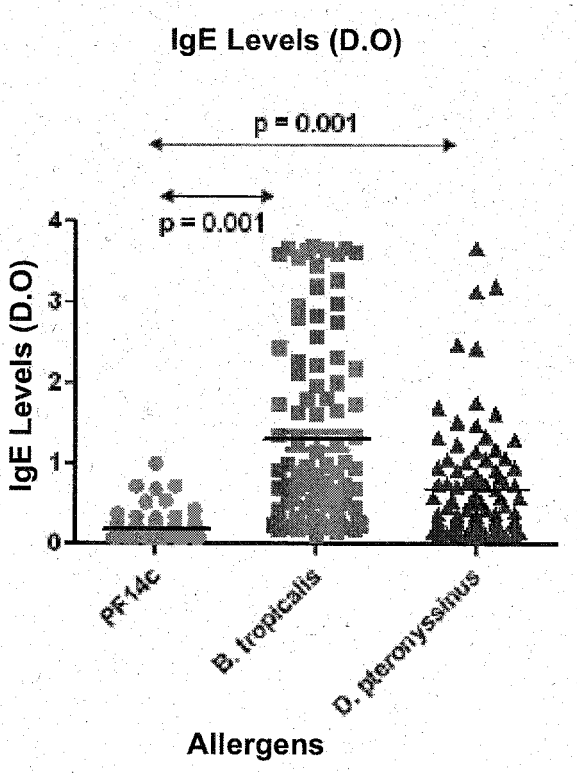

IgE Levels (D.O)
p = 0.001
p = 0.001
IgE Levels (D.O)
4
3
2
1
0
PF14c
B. tropicalis
D. pteronyssinus
Allergens

FIG. 4

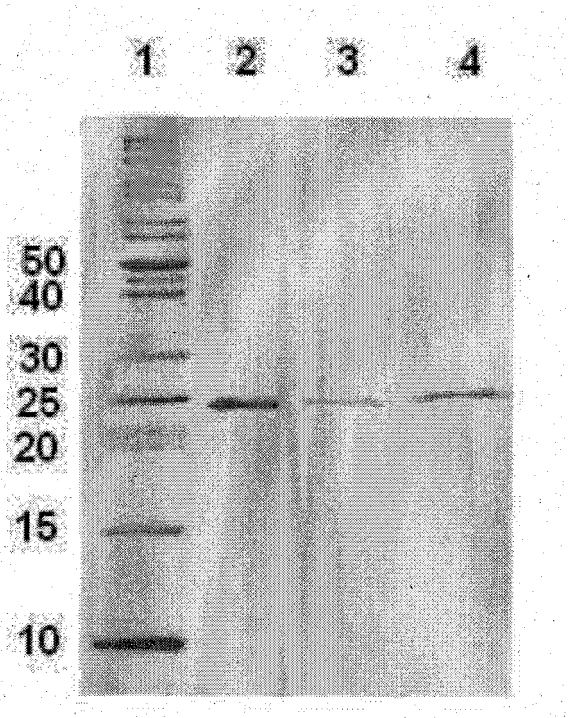


FIG. 5

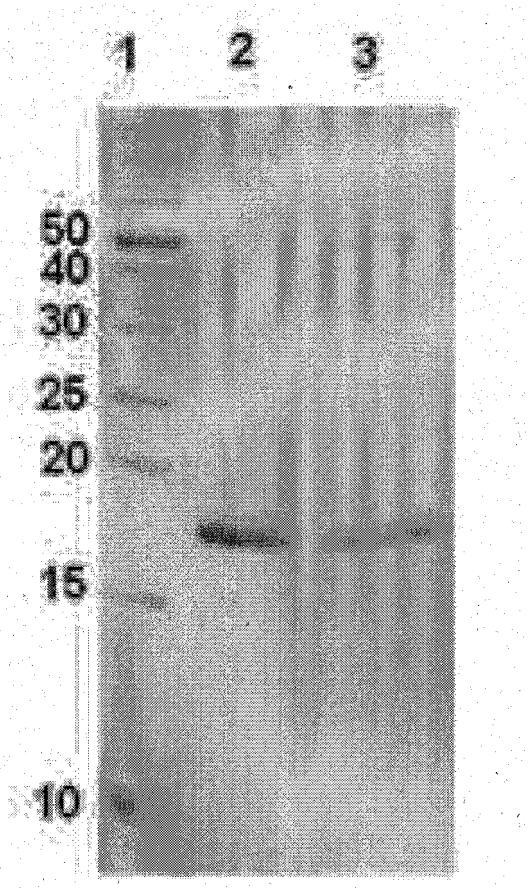


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/054994

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K, A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, INVENES, WPI, MEDLINE, BIOSIS, EMBASE, INSPEC, COMPDX, NPL, XPESP, XPESP2, TXTUS0, TXTUS1, TXTUS2, TXTUS3, TXTUS4, TXTEP1, TXTGB1, TXTWO1, TXTEPF, TXTWOF, TXTFR1, TXTCHF, TXTBEF, TXTCAF, UNIPROTKB, EPOP, JPOP, KPOP, USPOP, UNIPARC, INTACT, IMGTHLAP, IPDKIRP, DGENE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2007/065468 A1 (CHUA, KAW Y. ET AL.) 22/03/2007, the whole document.	1-9
A	HALES, B. J., MARTIN, A. C., PEARCE, L. J. et al. IgE and IgG anti-house dust mite specificities in allergic disease. Journal of Allergy and Clinical Immunology. August 2006, Vol. 118, Nº 2, pages: 361 - 367. ISSN 0091-6749. <Doi:10.1016/j.jaci.2006.04.001>	1-9
A	WO 2007/140505 A2 (BIOMAY AG) 13/12/2007, SEQ. ID. NO. 92, 99; example 25.	1-9

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance.
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure use, exhibition, or other means.
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other documents , such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30/04/2013	**(08/05/2013)**
Name and mailing address of the ISA/ OFICINA ESPAÑOLA DE PATENTES Y MARCAS Paseo de la Castellana, 75 - 28071 Madrid (España) Facsimile No.: 91 349 53 04	Authorized officer E. Relaño Reyes Telephone No. 91 3498504

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/054994

C (continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of documents, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JIMENEZ, S., PUERTA, L., MENDOZA, D. et al. IgE antibody responses to recombinant allergens of *Blomia tropicalis* and *Dermatophagoides pteronyssinus* in a tropical environment. Allergy and Clinical Immunology International. November 2007, Vol. 19, Nº 6, pages 233 - 238. ISSN 0838-1925 <Doi:10.1027/0838-1925.19.6.233> (abstract) EMBASE database. [retrieved the 30.04.2013] Retrieved from EPOQUE; Accession number: EMB-2008153247	1-7
A	ZAKZUK, J. FERNÁNDEZ-CALDAS, L., CARABALLO, L. et al. Evaluation of IgE responses against Blo t 8, a Glutathione S Transferase (GST) from *Blomia tropicalis* (Bt) mite. Journal of Allergy and Clinical Immunology. February 2010, Vol. 125, Nº 2, pages AB6. ISSN 0091-6749. <Doi:10.1016/j.jaci.2009.12.055>	1-7

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2012/054994

CLASSIFICATION OF SUBJECT MATTER

C07K14/435 (2006.01)
A61K39/35 (2006.01)
A61P37/08 (2006.01)

Form PCT/ISA/210 (extra sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/IB2012/054994

Patent document cited in the search report	Publication date	Patent family member(s)	Publication date
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		BRPI 0712421 A2	19.06.2012
		AU 2007257308 A1	13.12.2007
		AT 503690 A1	15.12.2007

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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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- US 20080274059 A **[0009] [0012]**
- US 20110052640 A **[0009] [0013]**
- US 20070065468 A **[0009] [0014]**
- US 20060233839 A **[0009] [0015]**
- US 20090130130 A **[0009] [0016]**
- WO 2007140505 A **[0009] [0017]**

Non-patent literature cited in the description

- **SAMBROOK ; RUSSEL.** The Condensed Protocols from Molecular Cloning: ALaboratory Manual. CSHL Press **[0047]**